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DNA METHYLATION, INDUCED BY BETA-CAROTENE AND ARACHIDONIC ACID, PLAYS A REGULATORY ROLE IN THE PRO-ANGIOGENIC VEGF-RECEPTOR (KDR) GENE EXPRESSION IN ENDOTHELIAL CELLS

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DNA methylation is a potent regulator of gene expression. The influence of beta-carotene (BC) and arachidonic acid (AA) on angiogenesis - a new blood vessel formation, was reported. The tyrosine kinase VEGFR-2 receptor (KDR) activation by vascular endothelial growth factor is one of the main angiogenic mechanisms. This study was aimed to investigate a possible role of CpG island methylation on regulation of the pro-angiogenic *KDR* gene expression after incubation of human endothelial cells with BC and/or AA. Methods: Human umbilical vein endothelial cells (HUVEC) were incubated with BC (1-10 μM) and/or 3 μM AA for 24 hours. The CpG island methylation was quantified using the COBRA method and restriction enzymes' digestion (NewEngland BioLabs). Intracellular protein concentrations were determined by Western blot analysis using the specific antibodies (Santa Cruz). Results: Incubation with BC and AA, decreased methylation of the *KDR* promoter region. These results well-correlated with the detected, by qRT-PCR, up-regulation of *KDR* gene expression by BC ($p=0.035$) as well as by AA. Incubation with BC ($p=0.02$) and AA ($p=0.0014$) increased the *KDR* protein levels in HUVECs. Conclusion: The changes in CpG island methylation of the *KDR* the pro-angiogenic gene promoter, represents one of the mechanisms involved in regulation of angiogenic response by BC and AA.

Key words: *angiogenesis, beta-carotene, vascular endothelial growth factor receptor, DNA methylation, HUVEC*

INTRODUCTION

DNA methylation is a potent regulator of gene expression (1). This mechanism plays a crucial role in both physiology *i.e.* fetal development, tissue remodelling, as well as pathology like, cardiovascular diseases, oncogenesis and metastasis, by regulation of the tumour angiogenesis (2-4).

Angiogenesis is a process of formation of new blood vessels from pre-existing capillaries and plays a crucial role in pathological events such as inflammation, diabetic retinopathy and tumour development (5). A number of growth factors (VEGF, bFGF, TGFβ and others), cell/matrix (integrins) and cell/cell (VE-cadherins, catenins, endoglins) interactions, as well as environmental factors (shear stress, oxygen supply and others) regulate the most important steps (detachment, proliferation, migration, differentiation and apoptosis) of angiogenesis (6, 7). The tyrosine kinase VEGFR-2 receptor (KDR) activated by vascular endothelial growth factor-A, is one of the main pro-angiogenic mechanisms (7).

Many environmental factors such as ultra violet light, free radicals, temperature, and diet components influence the regulatory processes of gene expression (8). One of the intensively investigated antioxidants is beta-carotene (BC), which was shown to reach the target cells in a non-metabolised form in humans (9). Some clinical trials, undertaken to test the efficacy of BC supplementation for the prevention of atherosclerosis or cancer, have revealed that long-term and high dosage BC administration increased risk of lung cancer, especially in smokers (10, 11). BC compound demonstrated pro-angiogenic activity, which was reported in the presence of the tissue inflammation or hypoxia (12). Our previous publications also describe BC pro-angiogenic role by demonstrating its pro-chemotactic activity in the endothelial cells (13, 14).

It has been shown that fatty acids increase cellular uptake of BC (15) and that a high fat diet, especially the metabolites of arachidonic acid (AA), participate in carcinogenesis (16). Arachidonic acid metabolites are also involved in immuno-modulatory reaction, regulation of angiogenesis and thrombosis (17). The recent publication reported that the regulatory role of AA on early phases of angiogenesis, such as proliferation and tubulogenesis, were related to the AA-activated endothelial cell calcium Ca^{2+} elevation (17).

The presented study was undertaken to investigate a possible role of the DNA (CpG) island methylation (1) in the regulation of expression of angiogenesis-controlling *KDR* gene after incubation of the endothelial cells with non-toxic amounts of BC and AA.

MATERIALS AND METHODS

Cell line incubation and biological effects

The primary endothelial cells, human umbilical vein endothelial cells (HUVECs), were isolated and cultured according

Table 1. Microarray analysis of global gene expression in HUVEC.
Changes in relative gene expression were calculated versus control (THF/EtOH solvent). Only spots with significant differences in signal intensity (more than 1.4-fold and only when $p \leq 0.05$) were included in the analysis. Using the described criteria we identified 838 genes, whose expression changed in response to the stimulation with BC while the expression of 644 genes was regulated by AA (13, 14). Generally β-carotene and arachidonic acid activated expression of genes connected with cell growth, adhesion, cell-cell signalling, chemotaxis, when inhibited genes connected with apoptosis.
↑up-regulation, ↓down-regulation, NC - no change

name of gene/ pathway	BC	AA
angiogenesis		
CCL2	↑	↑
angiopoietin 2	↑	↓
NOS- 3	↑	↑
KDR	↑	↑
integrins	↑	**NC**
metalloproteinases	↑	**NC**
cadherins	↓	**NC**
catenins	↓	↓
chemotactic activity		
CXCR4	↑	↑
IL-8	↑	**NC**
proliferation/differentiation		
WNT signalling	↓	↓
MAPK	↓	↓

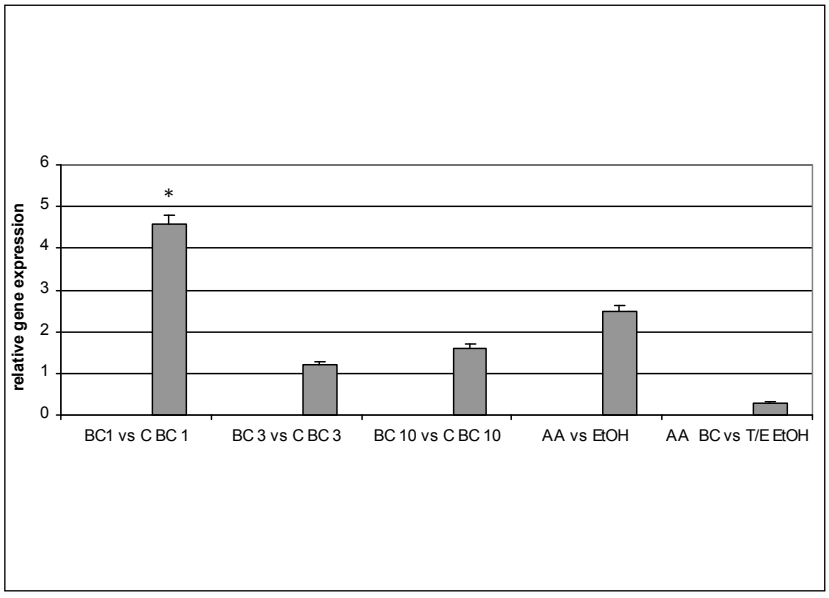

Fig. 1. Expression of *KDR* gene verified by the quantitative real-time PCR in HUVECs, after incubation with beta-carotene (BC) and arachidonic acid (AA) (Results are presented as mean values, obtained from three separate experiments measured in duplicates, +/-SD) significance (*) set at: $p<0.05$. The expression rates were calculated as the normalized CT difference between a control probe and sample with the adjustment for the amplification efficiency relative to the expression level of the housekeeping gene *GAPDH*. BC 1- beta-carotene at 1 μM concentration, C BC1 - control for 1 μM BC, BC 3- beta- carotene at 3 μM concentration, C BC 3 - control for 3 μM BC, BC 10- beta- carotene at 10 μM concentration, C BC 10 - control for 10 μM BC, AA - arachidonic acid, EtOH - control with ethanol (solvent) for AA, AA BC - co-incubation with BC and AA, T/E EtOH- control with THF and ethanol for AA with BC

to the protocol described previously (13, 14). Endothelial cells were incubated with non-toxic amounts of BC (1-10 μM), AA (3 μM) (Sigma), or both BC (3 μM) and AA (3 μM) for 24 hours in 37°C and 5% of CO_2 (Jouan IG 150 incubator). The uptake of BC was measured by HPLC (11). Migration of HUVECs was performed using the Boyden's Chamber assay, according to the protocol described previously (13, 14).

Analysis of gene expression

Total RNA was isolated from the endothelial cells by the guanidine thiocyanate-caesium chloride method using Trizol (Invitrogen Life Technologies) and purified using SV total RNA Isolation System Kit (Promega) (13). Global gene expression was assessed by a microarray analysis (Affymetrix HG-U133a, 14 500 genes), *Table 1* (13, 14). The changes in the BC or AA angiogenesis-related gene expression that were greater than 1.4 fold were confirmed by a quantitative real-time PCR (qRT-PCR) method using *GAPDH* as the reference gene, QuantiTect SYBR Green PCR (Qiagen), DNA Engine Opticon II (MJ Research) (14).

Analysis of DNA methylation status

Genomic DNA was isolated from the endothelial cells using a DNA isolation Mini Kit, Qiagen. CpG island methylation in genomic DNA was quantified with the Combined Bisulphite Restriction Analysis (COBRA) method (18). PCR with the specific primers amplifying promoter region of 18 genes involved in the angiogenesis regulation process, was performed according to the manufacturer protocol (HotStarTaq Master Mix Kit, Qiagen), and

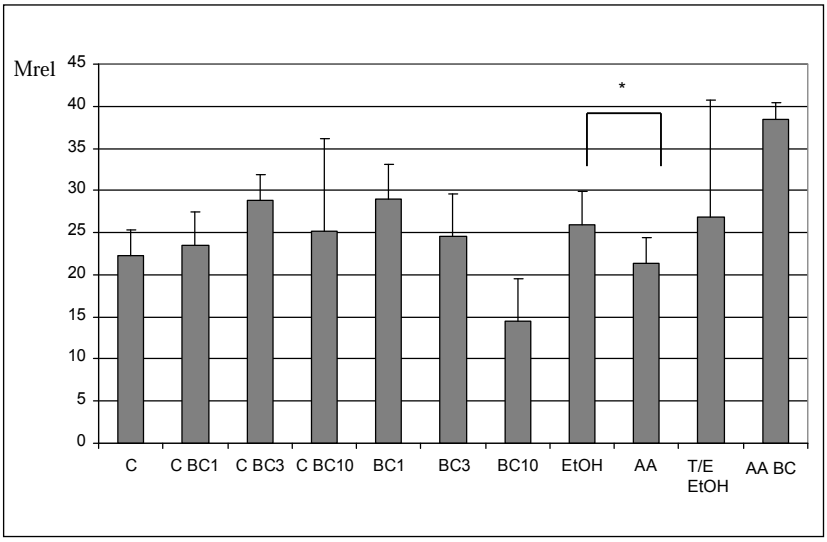

Fig. 2. Results of CpG island methylation of the *KDR* gene promoter after incubation with BC or/and AA. The results are presented as mean values +/-SD from pooled DNA from three independent experiments carried out in duplicate, *- $p<0.05$. Mrel - Relative methylation level, obtained from densitometry readings of PCR or digestion bands, and calculated according to the formula Mrel = M/(U+M) (Methylated or Unmethylated) (Muhlisch J, 2006).
BC 1- beta- carotene at 1 μM concentration, C BC1 - control for 1 μM BC, BC 3- beta- carotene at 3 μM concentration, C BC 3 - control for 3 μM BC, BC 10- beta- carotene at 10 μM concentration, C BC 10 - control for 10 μM BC, AA - arachidonic acid, EtOH - control with ethanol (solvent) for AA, AA BC - co-incubation with BC and AA, T/E EtOH- control with THF and ethanol for AA with BC

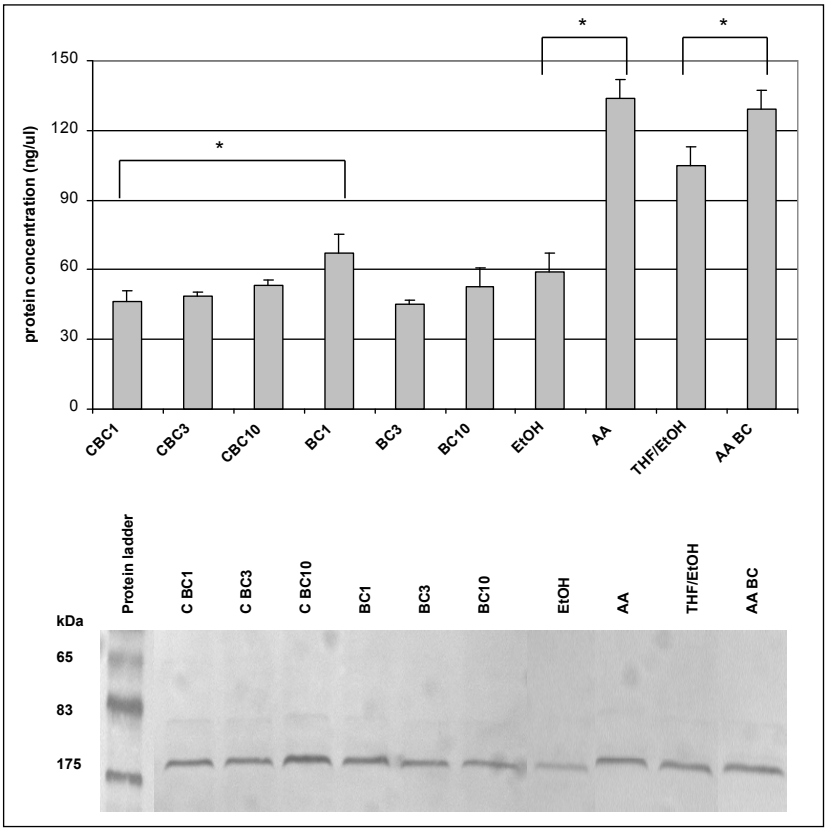

Fig. 3. Protein level of the VEGF receptor 2 (KDR) in HUVEC after incubation with BC and AA (results are shown as mean values, derived from three separate experiments, +/-SD) significance (*) set at: $p<0.05$.
BC 1- beta- carotene at 1 μM concentration, C BC1 - control for 1 μM BC, BC 3- beta- carotene at 3 μM concentration, C BC 3 - control for 3 μM BC, BC 10- beta- carotene at 10 μM concentration, C BC 10 - control for 10 μM BC, AA - arachidonic acid, EtOH - control with ethanol (solvent) for AA, AA BC - co-incubation with BC and AA, T/E EtOH- control with THF and ethanol for AA with BC

the PCR products were digested by specific restriction enzymes (NewEngland BioLabs). The results were analysed using UVISoft UVIBand Window Application software. Global methylation analysis was performed using the cytosine extension assay (19).

Measurement of KDR protein level (Western blot)

Protein concentrations in samples were estimated using Bradford method. Immunoblot analyses were performed according to Laemmli (20). The immunoblotted proteins were detected by a commercial Western blot kits with the use of primary antibodies specific for human VEGF receptor 2 (KDR) (150 kDa) and β-actin that served as a reference protein (SantaCruz). The secondary antibodies included alkaline phosphatase-conjugated goat anti-mouse antibody (Sigma). The protein band was visualized by bromochoroindolyl phosphatase/nitro blue tetrazolium (BCIP/NBT) substrate, which generates black-purple precipitate at the site of the enzyme binding. The protein bands obtained from densitometry readings were analysed using UVISoft UVIBand Window Application software.

Statistical analysis

Data were analysed by one-way ANOVA and unpaired t-test for comparisons of quantitative variables. The cut off for statistical significance was set at $p<0.05$. The statistical analysis was performed with Statistica 6 for Windows from Statsoft.

RESULTS

Our previous paper reported that the presence of BC (3 μM) in the cell culture medium for 24 hour resulted in a four-fold increase of HUVEC migration, an important step for angiogenesis (13).

In this study, we demonstrate that BC (1 μM) significantly induced the VEGF receptor gene (*KDR*) expression in HUVECs (p=0.035) as measured by the qRT- PCR method (*Fig. 1*). The incubation of HUVECs with 3 μM AA also resulted in up-regulation of the *KDR* gene expression but the changes did not reach statistical significance (*Fig. 1*).

The tendency to down-regulation of global DNA-methylation in HUVECs incubated with 1 μM BC and especially 3 μM AA was previously reported (19). In this study, were able to demonstrate decreased methylation of the *KDR* promoter after incubation of the cells with higher concentrations of BC (3 μM and 10 μM), however only incubation with 3 μM AA resulted in the change of the promoter methylation level that reached statistical significance (p=0.048) (*Fig. 2*).

The significantly increased KDR protein concentrations in HUVECs after incubation with 1 μM BC (p=0.02) as well as with the mixture of 3 μM BC and 3 μM AA (p=0.013) was detected (*Fig. 3*). The incubation with 3 μM AA caused the most prominent up-regulation of VEGF receptor 2 protein level in the investigated cells (p=0.0014) (*Fig. 3*).

DISCUSSION

The presented study demonstrated that the up-regulation of *KDR*, the pro-angiogenic VEGF receptor gene expression by BC as well as by AA in HUVECs is related to the hypometylation of the KDR promoter region. One of the epigenetic regulatory mechanisms of the gene expression is DNA CpG island methylation (22). Aberrant methylation of gene promoters has gained increasing importance due to its role in regulation of oncogenesis, progenitor cell differentiation, silencing of the protooncogenes as well as up- regulation of the tumour suppressor genes' expression (22, 23).

In our previously reported analysis of the group of 18 genes, which selection was based on the microarray results and qRT-PCR (13), pointed that changes in DNA methylation was detected in the promoter regions of such genes as: integrin 3, connexin 43, MMP-2, laminin, Notch4 and VCAM1 (the COBRA analysis). The moderate down-regulation of methylation status was found in the promoter regions of 8 out of 18 selected genes analysed in HUVECs after incubation with the physiological concentrations of BC and/or AA. The results, which are in agreement with the earlier reports (12), suggests that the pro-angiogenic properties of BC might be related to differentiation, activation of chemotaxis and homing of endothelial cells (13).

In the presented study, we have documented that incubation both with BC as well as with AA decreases the HUVEC global DNA methylation as well as the methylation of the proangiogenic VEGF receptor- *KDR* gene promoter. This observation is in agreement with the previous reports pointing to the role of hypomethylation in regulation of gene expression (1-3) and may add to understanding to the activation of proandiogenic properties of the investigated compounds. The changes in methylation status of the *KDR* gene promoter region presented in this study, correlated well with the BC-induced up-regulation of *KDR* gene expression, measured by q-RT-PCR. Also, the Western blot results confirmed increase of the KDR protein synthesis that corresponds to the hypomethylation of the *KDR* DNA promoter region in HUVECs after incubation with BC.

The incubation of HUVECs with physiological, non-toxic concentrations of BC decreases the global DNA methylation (19). The above observations are in agreement with the up-regulation of *KDR* expression confirmed by qRT-PCR method and increase of VEGF receptor 2 protein concentration measured by Western blot. However, CpG island methylation in *KDR* promoter region did not demonstrate a concentration-dependent effect. These results may suggest the involvement of an additional process, such as participation of other co-activators/suppressors in the regulation of *KDR* gene expression regulated by the used BC concentrations.

The correlation between DNA methylation, gene expression and changes in the protein level was also observed in the presence of AA. Similarly to BC the incubation with AA caused the HUVEC down regulation of global as well as CpG islands' *KDR* gene promoter methylation, and was associated with the up-regulation of *KDR* gene expression and significant increase of protein level of this VEGF receptor . The above observations are in agreement with the other reported study performed mostly on cancer cell lines, in which DNA methylation affected the up-regulation of VEGF receptor gene expression (24).

Up to date the role of CpG island methylation in the regulation of expression of genes controlling the process of angiogenesis was analysed in a relatively low number of works, which were mostly performed on the different cancer cell lines (2). The presented study focused on the role of DNA methylation in the process of angiogenesis in the endothelial cell line. The obtained data suggest that even minute alterations of promoter methylation induced by arachidonic acid in cooperation with beta-carotene may lead to changes in expression of the endothelial cell *KDR* gene, which is important for cell chemotaxis, differentiation and angiogenesis - the processes important for oncogenesis as well as for carcinogenesis.

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Conflict of interests: None declared.

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